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METHOD FOR STABILIZING PROTEIN SOLUTIONS

Cross-Reference to Related Applications

5   This application is the National Stage of International Application No. PCT/JP2004/014919, filed on October 8, 2004, which claims the benefit of Japanese Patent Application Serial No. 2003-351410, filed on October 9, 2003.   The contents of both of the foregoing applications are hereby incorporated by reference in their entireties.

10 Technical Field
     The present invention relates to methods for stabilizing proteins at low temperature.

Background Art
     Many higher animals have five different classes of immunoglobulins, IgG, IgA, IgM,
15 IgD, and IgE.   Each immunoglobulin class differs in properties such as size, charge, amino acid composition, and sugar content.   Of these classes, IgM accounts for approximately 10% of all plasma immunoglobulins.   IgM is the major component of early antibodies produced against cell-membrane antigens, infectious microorganisms, or soluble antigens, which have a complex antigenicity.
20   Human IgMs usually have a pentameric structure.   Each of the five subunits constituting this pentameric structure has a four-stranded structure similar to that of IgG.   The amino acid sequence of the μ chain, which is the heavy chain of IgM, is different from that of the γ chain, which is the heavy chain of IgG.   The following differences can also be seen:
     • The μ chain has an extra constant domain than the γ chain.
25   • The μ chain has four more oligosaccharide chains than the γ chain.
     IgM has a polypeptide chain called the J chain, which is not found in IgG.   The J chain is considered to assist the association of μ chains prior to secretion of IgM from antibody producing cells.
     With advances in monoclonal antibody technology and recombinant DNA technology,
30 large-scale production of pure immunoglobulins has become possible in recent years. Furthermore, gene recombination techniques have enabled production of chimeric antibodies and humanized antibodies.   Chimeric antibodies are antibodies having a structure in which the variable regions have been replaced with variable regions derived from a different species.   For example, "chimeric antibodies" comprising variable regions of non-human antibodies and the
35 constant regions of human antibodies (Non-Patent Document 1/ Proc. Natl. Acad. Sci. U.S.A.,

(1984) 81:6851) are known. Also known are humanized antibodies in which the complementarity determining regions (CDR) of other animal species are transferred into human immunoglobulins (Non-Patent Document 2/ Nature (1986) 321:521).

Actual examples of antitumor antibodies are the anti-CD20 human chimeric antibody
5   Rituxan (IDEC), and the anti-HER2/neu humanized antibody Herceptin (Genentech), which have completed clinical trials and have already been approved. These antibodies are now commercially available. Antibody-dependent cellular cytotoxicity (hereinafter referred to as ADCC) activity and complement-dependent cytotoxicity (hereinafter referred to as CDC) activity are known as effector functions of IgG and IgM. Since IgM has a higher CDC activity
10   compared to IgG, it has an extremely high chance of becoming an anti-tumor antibody having CDC activity as its main effect. However, as described above, unlike IgG, IgM forms a multimer. Therefore, industrial scale production of recombinant IgM had been considered difficult.

IgM is also very unstable compared to IgG and has a low solubility. Therefore, the
15   production of a highly concentrated and stable IgM solution is difficult. For example, Cytotherapy, 2001, 3(3), 233-242 (Non-Patent Document 5) reports that, even when IgM had been stored at -20°C, precipitation and decrease of activity occurred upon thawing. Furthermore, according to the report, IgM easily aggregates and precipitates during storage. Arch. Pathol. Lab. Med., 1999, 123, 119-125 (Non-Patent Document 6) showed that among
20   precipitates called cryoprecipitations or low-temperature precipitations observed in human serum, Type I cryoglobulin, which produces a precipitate consisting of a single antibody component, is mainly IgM. IgM, in particular, readily undergoes cryoprecipitation, making it difficult to obtain a highly concentrated IgM solution at a low temperature. Most biopharmaceuticals are stored and distributed under refrigeration at around 4°C to ensure stability. Since some IgMs
25   cryoprecipitate at around 4°C, it is preferable that their cryoprecipitation is suppressed during drug formulation, storage, and distribution. Cryoprecipitation also occurs in IgM bulk drug substance production processes leading to formulation, during purification and concentration steps at low temperature, and during low-temperature storage between the multiple steps involved. This causes operational problems, and thus, it is preferable to suppress
30   cryoprecipitation even in these circumstances.

Various attempts have been made to stabilize IgM at low temperature. For example, Immunochemistry, 1978, 15, 171-187 (Non-Patent Document 3) discloses that cryoprecipitation of IgM takes place more readily with temperature decrease and concentration increase. It also discloses that cryoprecipitation takes place in the pH range of 5 to 10, and that this
35   cryoprecipitation can be avoided at extremely high pH or low pH. However, antibodies generally tend to undergo a deamidation reaction and aggregation at high pH, and denaturation

and aggregation at low pH.   Antibodies are generally known to be chemically and physically stable from pH5 to pH8, especially near pH5 to pH7.   It is therefore difficult to ensure a stability sufficient enough to withstand pharmaceutical use at extremely high pH or low pH.

Journal of Biological Chemistry, 1997, 252(22), 8002-8006 (Non-Patent Document 4)

5   examined the effect of various compounds on cryoprecipitation (solubility of IgM at low temperature), and discloses that cryoprecipitation decreases when sugars are added or salt concentration is increased.   However, this disclosure shows that for effective prevention of cryoprecipitation using any sugars or salts, the sugars or salts must be added at high concentrations of approximately 500 mM or higher.   When used as a pharmaceutical, it is

10   preferable to achieve such an effect at lower concentrations.

WO 91/18106 (Patent Document 1) discloses methods for preventing cryoprecipitation by changing the structure of sugar chains attached to IgM.   However, when sugar chains of antibodies are modified, in some cases, the binding activities of antibodies change.   Therefore, it is desirable to develop methods for suppressing cryoprecipitation without altering the structure

15   of antibodies, including their sugar chains.

Patent Document 1: WO 91/18106

Non-Patent Document 1: Proc. Natl. Acad. Sci. U.S.A, (1984) 81: 6851

Non-Patent Document 2: Nature (1986) 321: 521

20   Non-Patent Document 3: Immunochemistry, 1978, 15, 171-187

Non-Patent Document 4: Journal of Biological Chemistry, 1997, 252(22), 8002-8006

Non-Patent Document 5: Cytotherapy, 2001, 3(3), 233-242

Non-Patent Document 6: Arch. Pathol. Lab. Med., 1999, 123, 119-125

25   Disclosure of the Invention

The present invention was achieved in view of the above circumstances.   An objective of the present invention is to stabilize proteins in solution at low temperature.   More specifically, the present invention aims to stabilize proteins under conditions (such as pH and salt concentration) that withstand pharmaceutical use.

30   To solve the above-mentioned problems, the present inventors examined the use of a citric acid buffer as a pH buffer, in the pH range of 5 to 8 where antibodies are generally considered to be stable, as a method for suppressing cryoprecipitation of IgM at a pH range and salt concentration suitable for IgM storage.   As a result, the citric acid buffer was found to significantly suppress cryoprecipitation.   More specifically, the use of citric acid buffer

35   enhanced the solubility of IgM at low temperature, and enabled preparation of highly concentrated IgM solutions.   This effect of citric acid on IgM is caused by adjustment of the

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strength of protein-protein interactions such as ionic interactions, van der Waals interactions, and hydrogen bonds. Accordingly, in addition to IgM, this effect can be accomplished in various other proteins that show decreased solubility in aqueous solutions at low temperature.

Specifically, the present invention relates to methods for stabilizing proteins at low temperature; more specifically the present invention provides the following:

(1)   a method for stabilizing a protein at low temperature, wherein the method comprises adding a citric acid buffer to a solution comprising the protein;

(2)   the method of (1), wherein the protein is stabilized by suppressing cryoprecipitation;

(3)   the method of (1), wherein the protein is IgM; and

(4)   the method of (1), wherein pH of the solution comprising the protein is 5 to 8.

Brief Description of the Drawings

Fig. 1 is a set of photographs showing the effect of citric acid buffer on the stability of IgM at various concentrations at low temperature (4°C).

Fig. 2 shows the effect of citric acid buffer on the stability of 10 mg/mL IgM solutions at low temperature (1°C, 4°C, and 7°C).

Fig. 3 is a set of photographs showing the effect of citric acid buffer on the stability of 10 mg/mL IgM solutions at low temperature (4°C).

Fig. 4 shows the effect of citric acid buffer on the stability of 10 mg/mL IgM solutions at low temperature (4°C).

Detailed Description

In the present invention, the term "proteins" refers to compounds in which amino acids are linked to each other through peptide bonds. Any protein whose solubility in aqueous solutions is decreased at low temperatures is suitable for the present invention, examples being IgG and peanut agglutinin (PNA).

IgM is particularly preferred as a protein in the present invention. In the present invention, the term "IgM" refers to an immunoglobulin that comprises constant regions of the μ chain as the constant regions of the heavy chains, and forms a pentameric or hexameric structure. The origin of the variable regions constituting the IgM of the present invention is not limited. Therefore, in addition to a variable region derived from the μ chain, the IgM of the present invention may comprise a variable region derived from IgG, or a partial structure thereof. The partial structure of a variable region can comprise the framework and CDR. The "IgM" in the present invention refers to expression products of exogenous IgM genes introduced into cells for transformation.

Furthermore, the constant regions constituting the IgM of the present invention may be

derived from any animal species. That is, the IgM of the present invention comprises an IgM constant region derived from any type of animal species carrying an IgM-type immunoglobulin. When IgM is administered *in vivo*, at least its constant regions are preferably derived from an animal species same as the species to which the IgM is administered. Therefore, when the IgM

5 is administered to humans, at least its constant regions are preferably derived from humans. IgM composed of constant regions derived from humans, and variable regions derived from another animal species or another human, is called a chimeric antibody. A more preferable IgM for administration to humans is an IgM whose variable region framework is derived from humans, in addition to the constant regions. Human antibodies which have retained the

10 variable region framework structure, but only the CDR has been replaced with that of an antibody from another animal species are called humanized antibodies.

Cryoprecipitation of highly concentrated proteins can be suppressed by the present invention. Herein, "highly concentrated" refers to a concentration in solution higher than 1 mg/mL (for example, 5 mg/mL or more, 10 mg/mL or more, 20 mg/mL or more, or 25 mg/mL or

15 more).

"Citric acid buffers" that may be used in the present invention are not limited to buffers that utilize only citric acid as the pH buffering agent, and may comprise pH buffering agents such as phosphoric acid other than citric acid.

The concentration of citric acid buffer added to solutions is usually 1 mM to 500 mM,

20 preferably 5 mM to 100 mM, and more preferably 10 mM to 50 mM. The term "stabilization" in the present invention refers to suppressing the increase of cryoprecipitated proteins in solutions.

The stability of protein solutions can be determined, for example, from the cryoprecipitation increase suppression rate that can be derived from the following formula.

25

Suppression ratio of cryoprecipitation increase = (A-B)/A x 100

A: cryoprecipitate formation ratio in a highly concentrated IgM solution to which a citric acid buffer has not been added (Control)

B: cryoprecipitate formation ratio in a highly concentrated IgM solution to which a citric acid

30 buffer has been added (Test sample)

The solutions of the present invention have a cryoprecipitation increase suppression rate of preferably 10% or more, more preferably 30% or more, even more preferably 50% or more, and yet even more preferably 80% or more, after adding a citric buffer and keeping at 1°C for a

35 week.

The pH of the protein-comprising solutions of this invention can be adjusted to a value

at which proteins are stable, and specifically, pH5 to pH8 is preferable. Furthermore, the pH of the protein-comprising solutions of this invention can be adjusted to a value suitable for stable storage of the proteins, and specifically, pH5 to pH7 is preferable, while pH5 to pH6 is more preferable.

5   The dosage form of pharmaceutical formulations of the present invention is not particularly limited, and any discretionary dosage form is possible. Examples of the dosage form include a solution formulation and a lyophilized formulation. Examples of the solution formulations include formulations stored in a cold place, formulations stored at room temperature, and frozen formulations. There are no particular limitations on the administration

10   route for the pharmaceutical formulations of the present invention; any administration route can be used. The pharmaceutical formulations may thus be administered either orally or parenterally depending on the purpose of use.

      Specific dosage forms for parenteral administration include injections, and dosage forms for nasal administration, pulmonary administration, and transdermal administration. Systemic

15   or local injections can be carried out by intravenous injections, intramuscular injections, peritoneal injections, subcutaneous injections, or such.

      In addition to administering directly to patients as is, IgM stabilized by methods of the present invention can be administered as pharmaceutical agents formulated by well-known pharmaceutical methods. For example, the stabilized IgM can be used as sterile solutions

20   prepared with water or other pharmaceutically acceptable liquid, or as injections of suspensions. Furthermore, it may be formulated by, for example, appropriately combining with pharmaceutically acceptable carriers or media, such as sterilized water, saline, emulsifiers, suspending agents, surfactants, stabilizers, vehicles, and preservatives, and mixing them at a unit dosage form required for generally accepted pharmaceutical practice. The amount of active

25   ingredient in these formulations can be adjusted so that an appropriate dose within an indicated range can be acquired.

      Sterile compositions for injections can be formulated according to usual pharmaceutical practice using vehicles such as distilled water for injections. Examples of aqueous solutions used for injections include physiological saline and isotonic solutions comprising glucose and

30   other auxiliary agents. Specifically, the auxiliary agents may be D-sorbitol, D-mannose, D-mannitol, sodium chloride, and such. Suitable solubilizers may also be added to pharmaceutical compositions. For example, alcohols and non-ionic surfactants are preferred solubilizers. Specific examples of alcohols comprise ethanol, polyalcohols such as propylene glycol and polyethylene glycol. Examples of non-ionic surfactants may be Polysorbate80 or

35   HCO-50. Cationic surfactants such as benzalkonium chloride may also be used.

      Oily fluids may be, for example, sesame oil and soybean oil, and may be used together

with benzyl benzoate or benzyl alcohol as a solubilizer. Furthermore, buffers such as phosphate buffer and sodium acetate buffer, analgesic agents such as procain hydrochloride, stabilizers such as benzyl alcohol and phenol, and antioxidants may be combined. The prepared injections are usually loaded into suitable vials or ampules.

5   The administration dose of the pharmaceutical formulations can be appropriately selected according to the disease to be treated, and age and symptoms of the patient. For example, a single dose can be selected within the range of 0.0001 mg to 1,000 mg per 1 kg body weight. Alternatively, for example, the dose can be selected within the range of 0.001 to 100,000 mg/body of patient. However, doses of the pharmaceutical formulations of the present invention are not limited to these.

10   One can refer to WO 2002/096457 for the preparation of liquid formulations and such of the present invention.

        All prior art literature cited herein are incorporated herein by reference.

15   Examples

        Hereinafter, the present invention is specifically illustrated with reference to Examples, but it is not to be construed as being limited thereto.

[Example 1]

20   In the following Examples, recombinant anti-ganglioside GM3 human antibody produced in the Reference Example (hereinafter, referred to as "MABON-01") was used as the IgM. Highly concentrated MABON-01 solutions were produced at room temperature. The compositions of the solutions were as follows:

Citric acid buffer: 20 mM sodium citrate, 300 mM NaCl, pH5.5 (citric acid buffer)

25   Acetic acid buffer: 20 mM sodium acetate, 300 mM NaCl, pH5.5 (acetic acid buffer)

        IgM-comprising citric acid buffers and acetic acid buffers were named as shown in Table 1 for convenience according to the concentration of IgM.

Table 1

MABON-01	Acetic acid buffers	Citric acid buffers
50 mg/mL	A5	C5
33 mg/mL	A4	C4
25 mg/mL	A3	C3
17 mg/mL	A2	C2
8 mg/mL	A1	C1

30

These solutions stored at 4°C are shown in Fig. 1. Whereas cryoprecipitation was clearly observed in the acetic acid buffer A4 and A5 comprising highly concentrated MABON-01, cryoprecipitation was not observed in citric acid buffer solutions (C4 and C5) comprising the same concentration of MABON-01. This revealed that use of citric acid as the buffer enables preparation of highly concentrated solutions with no cryoprecipitation.

[Example 2]

An approximately 20 mg/mL solution of MABON-01 in a 20 mM sodium acetate, 300 mM NaCl, pH 6.0 solution was prepared at room temperature, and dialyzed at 4°C against 20 mM sodium citrate, 300 mM NaCl, pH5.5 (citric acid acid buffer), or 20 mM sodium acetate, 300 mM NaCl, pH6.0 (acetic acid buffer) using a dialyzer membrane EasySep (TOMY) to exchange the buffer. After warming to room temperature, the solutions were diluted using each corresponding buffer to prepare 10 mg/mL solutions. These solutions were placed in 0.5-mL PCR tubes, and stored for 26 days at 7°C, 4°C, or 1°C. Cryoprecipitate formation was then visually observed. After centrifugation, the MABON-01 concentrations in the obtained supernatants were determined by gel filtration chromatography. In the gel filtration chromatography, a G4000SW$_{XL}$ (TOSOH) column was used, and a 50 mM sodium phosphate, 500 mM KCl, pH7.4 solution was used as the mobile phase. The values of the sum of the aggregate peak area and the monomer peak area before and after cryoprecipitation as determined by gel filtration chromatography were compared, and the cryoprecipitate formation rate of MABON-01 was calculated.

Visually, cryoprecipitation was observed when the MABON-01 solution in acetic acid buffer was stored at 4°C and 1°C, but not in other solutions.

The cryoprecipitate formation ratio in each sample is shown in Fig. 2. In all buffer systems, the tendency was that the lower the temperature, the higher the level of precipitation. However, at all temperatures, the precipitation amount was lower in the citric acid buffer systems than in the acetic acid buffer systems, confirming a clear cryoprecipitation suppression effect due to the use of a citric acid buffer. Changing the buffer from 20 mM acetic acid buffer to 20 mM citric acid buffer showed that it is possible to store at lower temperatures without adding high concentrations of salts or such.

[Example 3]

An approximately 20 mg/mL solution of MABON-01 in a 20 mM sodium acetate, 300 mM NaCl, pH 6.0 solution was prepared at room temperature, and dialyzed at 4°C against 20 mM sodium citrate, 300 mM NaCl, pH5.0, pH5.5, or pH6.0 (citric acid acid buffer), or 20 mM sodium acetate, 300 mM NaCl, pH5.0, pH5.5, or pH6.0 (acetic acid buffer), using a dialyzer

membrane EasySep (TOMY), to exchange the buffer.   After heating to room temperature, the solutions were diluted using each corresponding buffer to prepare 10 mg/mL solutions.   These solutions were placed in 0.5-mL PCR tubes, and stored for 29 days at 4°C.   Cryoprecipitate formation was then visually observed.   After centrifugation, the MABON-01 concentrations in

5   the obtained supernatants were determined by gel filtration chromatography.   In the gel filtration chromatography, a G4000SW$_{XL}$ (TOSOH) column was used, and a 50 mM sodium phosphate, 500 mM KCl, pH7.4 solution was used as the mobile phase.   The values of the sum of the aggregate peak area and the monomer peak area before and after cryoprecipitation as determined by gel filtration chromatography were compared, and the cryoprecipitate formation

10   rate of MABON-01 was calculated.

Visual observations of the solutions are shown in Fig. 3.   Cryoprecipitation was observed in acetic acid buffers at pH5.5 and pH6.0, but no precipitation was observed in any other solutions.

The cryoprecipitate formation ratio is shown in Fig. 4.   Whereas the acetic acid buffer

15   system showed a curve that reaches a maximum at pH5.5, the citric acid buffer system had low level of precipitates and a particular tendency was not observed.   When comparisons were made within pH5.5 to pH6.0, which is the most suitable pH range for pharmaceutical preparations, changing from a 20 mM acetic acid buffer solution system to a 20 mM citric acid buffer system suppressed cryoprecipitation even when the pH was kept at the same value.

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[Reference Example 1]   Production of recombinant human antibodies against ganglioside GM3
1.1 Construction of anti-ganglioside GM3 human antibody heavy chain gene

A gene encoding the heavy chain of a human antibody that binds to ganglioside GM3 was amplified by RT-PCR using total RNAs extracted from human B cells transformed with

25   Epstein-Barr virus (hereinafter, denoted as anti-ganglioside GM3 human antibody-expressing B cells).

Total RNAs were extracted from 1 x 10^7 anti-ganglioside GM3 human antibody-expressing B cells using RNeasy Plant Mini Kit (QIAGEN).   Two oligonucleotides (LMH-f3 and LMH-r3) were designed based on the nucleotide sequence of anti-ganglioside

30   GM3 human antibody gene reported by Hoon et al. (Cancer Research 1993; 53: 5244-5250). LMH-f3 (SEQ ID NO: 7) was synthesized in the sense direction, and LMH-r3 (SEQ ID NO: 8) was synthesized in the antisense direction.   Using 1 µg of total RNAs, gene fragments were amplified separately for the 5' end and the 3' end by SMART RACE cDNA Amplification Kit (CLONTECH).   Synthetic oligonucleotides LMH-r3 and LMH-f3 were used for amplifying the

35   5' and 3' ends of the gene, respectively.   Reverse transcription reaction was carried out at 42°C for 1.5 hours.

The composition of the PCR reaction solution (50 μL) is shown below:

5 μL of 10x Advantage 2 PCR Buffer,

5 μL of 10x Universal Primer A Mix,

5   0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),

1μL of Advantage 2 Polymerase Mix,

            (All the above were from CLONTECH)

2.5 μL of reverse transcription product, and

10 pmol of synthetic oligonucleotide LMH-f3 or LMH-r3.

10

The reaction was carried out under the conditions of:

94°C (initial temperature) for 30 seconds,

5 cycles of 94°C for 5 seconds and 72°C for 3 minutes,

5 cycles of 94°C for 5 seconds, 70°C for 10 seconds, and 72°C for 3 minutes,

15   25 cycles of 94°C for 5 seconds, 68°C for 10 seconds, and 72°C for 3 minutes, and

finally 72°C for 7 minutes.

The PCR products were purified from agarose gel using QIAquick Gel Extraction Kit (QIAGEN), and then cloned into pGEM-T Easy vector (Promega). After sequencing, an

20   approximately 1.1 kbp fragment was obtained by digesting the vector comprising the 5' end of the gene using restriction enzymes ApaI (Takara Shuzo) and SacII (Takara Shuzo), while an approximately 1.1 kbp fragment was obtained by digesting the vector comprising the 3' end of the gene using restriction enzymes ApaI (Takara Shuzo) and NotI (Takara Shuzo). The fragments were then mixed, and cloned into pBluescript KS+ vector (TOYOBO) to obtain a

25   full-length anti-ganglioside GM3 human antibody heavy chain gene.

To clone into vectors for expression in animal cells, full-length gene fragments were amplified using synthetic oligonucleotides LMH-fxho and LMH-rsal. LMH-fxho (SEQ ID NO: 11) is a forward primer designed to hybridize to the 5' end of the anti-ganglioside GM3 human antibody heavy chain gene, and to comprise an XhoI restriction enzyme recognition sequence

30   and a Kozak sequence. LMH-rsal (SEQ ID NO: 12) is a reverse primer designed to hybridize to the 3' end of the anti-ganglioside GM3 human antibody heavy chain gene, and to comprise a SalI restriction enzyme recognition sequence.

The composition of the PCR reaction solution (50 μL) is shown below:

35   5 μL of 10x PCR Buffer,

1 mM MgSO$_4$,

0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),

1 unit of DNA polymerase KOD-Plus,

(All the above were from TOYOBO)

10 ng of pBluescript KS+ vector comprising the full-length anti-ganglioside GM3 human

5   antibody heavy chain gene, and

10 pmol of synthetic oligonucleotides LMH-fxho and LMH-rsal.

The reaction was carried out under conditions of:

94°C (initial temperature) for 2 minutes,

10   30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 2 minutes, and

finally 72°C for 5 minutes.

The amplified gene fragment was cloned by digesting with the XhoI restriction enzyme

(Takara Shuzo) and the SalI restriction enzyme (Takara Shuzo), then purifying using QIAquick

15   PCR Purification Kit (QIAGEN), and linking to the XhoI restriction enzyme site of pUCAG.

This pUCAG vector is obtained by: linking the 2.6 kbp fragment obtained by digesting pCXN

(Niwa *et al.*, Gene 1991; 108: 193-200) using the BamHI restriction enzyme to the BamHI

restriction enzyme site of pUC19 vector (TOYOBO).   The obtained plasmid was named

pUCAG/L612H.   The nucleotide sequence and amino acid sequence of the anti-ganglioside

20   GM3 human antibody heavy chain in this plasmid are shown in SEQ ID NOs: 1 and 2,

respectively.

1.2 Construction of anti-ganglioside GM3 human antibody light chain gene

A gene encoding the light chain of anti-ganglioside GM3 human antibody was amplified

25   by RT-PCR using total RNAs extracted from the anti-ganglioside GM3 human

antibody-expressing B cells.   The total RNAs were extracted from the anti-ganglioside GM3

human antibody-expressing B cells in a manner similar to that mentioned above.   Two

oligonucleotides (LML-f1 and LML-r1) were designed based on the nucleotide sequence of

anti-ganglioside GM3 human antibody gene reported by Hoon *et al.* (Cancer Research 1993; 53:

30   5244-5250).   LML-f1 (SEQ ID NO: 9) and LML-r1 (SEQ ID NO: 10) were synthesized in the

sense and antisense directions, respectively.

Using 1 µg of total RNAs, gene fragments were amplified separately for the 5' end and

the 3' end by the SMART RACE cDNA Amplification Kit (CLONTECH).   Synthetic

oligonucleotides LML-r1 and LML-f1 were used for amplifying the 5' and 3' ends of the gene,

35   respectively.   Reverse transcription reaction was carried out at 42°C for 1.5 hours.

The composition of the PCR reaction solution (50 μL) is shown below:

5 μL of 10x Advantage 2 PCR Buffer,

5 μL of 10x Universal Primer A Mix,

0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),

5   1 μL of Advantage 2 Polymerase Mix,

(All the above were from CLONTECH)

2.5 μL of reverse transcription product, and

10 pmol of synthetic oligonucleotide LML-f1 or LML-r1

10   The reaction was carried out under conditions of:

94°C (initial temperature) for 30 seconds,

5 cycles of 94°C for 5 seconds and 72°C for 3 minutes,

5 cycles of 94°C for 5 seconds, 70°C for 10 seconds, and 72°C for 3 minutes,

25 cycles of 94°C for 5 seconds, 68°C for 10 seconds, and 72°C for 3 minutes, and

15   finally 72°C for 7 minutes.

PCR product was purified from the agarose gel using QIAquick Gel Extraction Kit (QIAGEN), and then cloned into pGEM-T Easy vector (Promega). After sequencing, an approximately 0.7 kbp fragment was obtained by digesting the vector comprising the 5' end of

20   the gene using the EcoRI restriction enzyme (Takara Shuzo), while an approximately 0.9 kbp fragment was obtained by digesting the vector comprising the 3' end of the gene using the EcoRI restriction enzyme (Takara Shuzo). The two fragments were mixed, and used to amplify the full-length gene fragment using synthetic oligonucleotides LML-feco and LML-mot. LML-feco (SEQ ID NO: 13) is a forward primer, and was designed to hybridize to the 5' end of

25   the anti-ganglioside GM3 human antibody light chain gene, and to comprise an EcoRI restriction enzyme recognition sequence and a Kozak sequence. LML-mot (SEQ ID NO: 14) is a reverse primer, and was designed to hybridize to the 3' end of the anti-ganglioside GM3 human antibody light chain gene, and to comprise a NotI restriction enzyme recognition sequence.

30   The composition of the PCR reaction solution (50 μL) is shown below:

5 μL of 10x PCR Buffer,

1 mM MgSO₄,

0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),

1 unit of DNA polymerase KOD-Plus,

35   (All the above were from TOYOBO)

5'-end gene fragment,

3'-end gene fragment, and

10 pmol of synthetic oligonucleotides LML-feco and LML-rnot.

The reaction was carried out under conditions of:

5   94°C (initial temperature) for 2 minutes,

30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 2 minutes, and

finally 72°C for 5 minutes.

The amplified gene fragment was cloned by digesting with the EcoRI restriction

10   enzyme (Takara Shuzo) and the NotI restriction enzyme (Takara Shuzo), then purifying using

QIAquick PCR Purification Kit (QIAGEN) and linking to the EcoRI and NotI restriction enzyme

cleavage sites of pCXND3.

The pCXND3 vector was constructed as follows: DHFR-ΔE-rvH-PM1-f (see WO

92/19759) was digested at the EcoRI/SmaI restriction enzyme site to separate their antibody

15   heavy chain gene and vector region.   Only the vector portion was then collected, into which the

EcoRI-NotI-BamHI adaptor (Takara Shuzo) was cloned.   This vector was named pCHOI.

A vector in which the DHFR gene expression site of pCHOI is cloned into the HindIII

restriction enzyme site of pCXN (Niwa *et al.*, Gene 1991; 108:193-200) was named pCXND3.

Furthermore, the light-chain gene fragment was cloned into pCXND3 and the obtained plasmid

20   was named pCXND3/L612L.   The nucleotide sequence and amino acid sequence of

anti-ganglioside GM3 human antibody light chain in this plasmid are shown in SEQ ID NOs: 3

and 4, respectively.

1.3 Construction of the anti-ganglioside GM3 human antibody expression vector

25   To produce the anti-ganglioside GM3 human antibody expression vector,

pUCAG/L612H was digested with the HindIII restriction enzyme (Takara Shuzo), and the

resulting an approximately 4.0 kbp fragment was linked to the HindIII restriction enzyme

cleavage site of pCXND3/l612L.   The obtained plasmid was named pCXND3/L612IgM.   This

plasmid expresses the neomycin-resistance gene, DHFR gene, and anti-ganglioside GM3 human

30   antibody gene in animal cells.

1.4 Construction of anti-ganglioside GM3 human antibody J-chain gene and expression vector

A gene encoding the J chain of anti-ganglioside GM3 human antibody was amplified by

RT-PCR using total RNAs extracted from anti-ganglioside GM3 human antibody-expressing B

35   cells.   Total RNAs were extracted from anti-ganglioside GM3 human antibody-expressing B

cells in a manner similar to that mentioned above.   Two oligonucleotides (J-f1 and J-r1) were

designed and synthesized based on the nucleotide sequence of the human antibody J chain gene registered in GenBank (GenBank accession number: M12759). J-fl (SEQ ID NO: 15) hybridizes to human antibody J chain gene Exon 3 in the sense direction, and J-r1 (SEQ ID NO: 16) hybridizes to the human antibody J chain gene Exon 4 in the antisense direction.

5   Using 1 µg of total RNAs, gene fragments were amplified separately for the 5' end and the 3' end by the SMART RACE cDNA Amplification Kit (CLONTECH). Synthetic oligonucleotides J-r1 and J-fl were used for amplifying the 5' and 3' ends of the gene, respectively. Reverse transcription reaction was carried out at 42°C for 1.5 hours.

10   The composition of the PCR reaction solution (50 µL) is shown below:

       5 µL of 10x Advantage 2 PCR Buffer,

       5 µL of 10x Universal Primer A Mix,

       0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),

       1 µL of Advantage 2 Polymerase Mix,

15   (All the above were all from CLONTECH)

       2.5 µL of reverse transcription product, and

       10 pmol of synthetic oligonucleotide J-fl or J-r1

The reaction was carried out under conditions of:

20   94°C (initial temperature) for 30 seconds,

       5 cycles of 94°C for 5 seconds and 72°C for 3 minutes,

       5 cycles of 94°C for 5 seconds, 70°C for 10 seconds, and 72°C for 3 minutes,

       25 cycles of 94°C for 5 seconds, 68°C for 10 seconds, and 72°C for 3 minutes, and

       finally 72°C for 7 minutes.

25

       PCR product was purified from the agarose gel using QIAquick Gel Extraction Kit (QIAGEN), and then cloned into pGEM-T Easy vector (Promega).

       After sequencing, an approximately 0.5 kbp fragment was obtained by digesting the vector comprising the 5' end of the gene using the EcoRI restriction enzyme (Takara Shuzo), and

30   an approximately 1.0 kbp fragment was obtained by digesting the vector comprising the 3' end of the gene using the EcoRI restriction enzyme (Takara Shuzo). The two fragments were mixed, and used to amplify the full-length gene fragment using synthetic oligonucleotides J-feco and J-rxba.

       J-feco (SEQ ID NO: 17) is a forward primer designed to hybridize to the 5' end of the

35   anti-ganglioside GM3 human antibody J chain gene, and to comprise an EcoRI restriction enzyme recognition sequence and a Kozak sequence. J-rxba (SEQ ID NO: 18) is a reverse

primer designed to hybridize to the 3' end of the anti-ganglioside GM3 human antibody J chain gene, and to comprise an XbaI restriction enzyme recognition sequence.

The composition of the PCR reaction solution (50 μL) is shown below:

5   5 μL of 10x PCR Buffer,

1 mM MgSO$_4$,

0.2 mM dNTPs (dATP, dGTP, dCTP, and dTTP),

1 unit of DNA polymerase KOD-Plus,

(the above-mentioned ingredients were all from TOYOBO)

10   5'-end gene fragment,

3'-end gene fragment, and

10 pmol of synthetic oligonucleotides LML-feco and LML-rxba

The reaction was carried out under conditions of:

15   94°C (initial temperature) for 2 minutes,

30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 2 minutes, and

finally 72°C for 5 minutes.

The amplified gene fragment was cloned by digesting with the EcoRI restriction

20   enzyme (Takara Shuzo) and the XbaI restriction enzyme (Takara Shuzo), then purifying using QIAquick PCR Purification Kit (QIAGEN), and linking to the EcoRI and XbaI restriction enzyme cleavage sites of pCOSII-Zeo.

This pCOSII-Zeo vector is obtained by removing the DHFR gene expression site of pCHOI, and cloning the Zeocin-resistant gene expression site thereto.   The obtained plasmid

25   was named pCOSII-Zeo/ J chain.   The nucleotide sequence and amino acid sequence of anti-ganglioside GM3 human antibody J chain in this plasmid are shown in SEQ ID NOs: 5 and 6, respectively.

1.5 Expression of anti-ganglioside GM3 human antibody using animal cells

30   Stable expression cell lines derived from CHO cells (DG44 line) were produced as described below.   Genes were introduced by electroporation using Gene Pulser II (BioRad).

Introduction of genes to obtain cell lines that do not express the J chain is described below.   0.75 mL of CHO cells suspended in PBS (1 x 10^7 cells/mL) was mixed with anti-ganglioside GM3 human antibody expression vector pCXND3/L612IgM (25 μg), cooled on

35   ice for 10 minutes, transferred to a cuvette, and then pulsed at 1.5 kV and 25 μFD.

After a recovery period of 10 minutes at room temperature, the electroporated cells were

suspended in 40 mL of CHO-S-SFMII medium (Invitrogen) comprising 1x HT Supplement (Invitrogen). A 50-fold diluted solution was further prepared using the same medium, and then aliquoted at 100 µL/well into a 96-well culture plate. After incubation for 24 hours in a CO_2 incubator (5% CO_2), Geneticin (Invitrogen) was added to the wells at 0.5 mg/mL and cultured for 2 weeks.

The IgM levels in the culture supernatants of wells in which colonies of Geneticin-resistant transformants were found were measured by the concentration assay described in Reference Example 1.6. Cell lines highly expressing the anti-ganglioside GM3 human antibodies were successively expanded to obtain anti-ganglioside GM3 human antibody-expressing stable cell lines CA02, CA15, CA19, CA20, and CA24.

Introduction of genes to obtain cell lines expressing the J chain is described below. 0.75 mL of CHO cells suspended in PBS (1×10^7 cells/mL) was mixed with anti-ganglioside GM3 human antibody expression vector pCXND3/L612IgM (25 µg) and J chain expression vector pCOSII-Zeo/J chain (20 µg), cooled on ice for 10 minutes, transferred to a cuvette, and then pulsed at 1.5 kV and 25 µFD.

After recovered for 10 minutes at room temperature, the electroporated cells were suspended in 40 mL of CHO-S-SFMII medium (Invitrogen) comprising 1x HT Supplement (Invitrogen).

A 50-fold diluted solution was further prepared using the same medium and aliquoted at 100 µL/well into a 96-well culture plate. After incubation for 24 hours in a CO_2 incubator (5% CO_2), 0.5 mg/mL Geneticin (Invitrogen) and 0.6 mg/mL Zeocin (Invitrogen) were added to wells, and cultured for 2 weeks. The IgM levels in the culture supernatants of wells in which colonies of Geneticin- and Zeocin-resistant transformants were found were measured by the concentration assay described in Reference Example 1.6. Cell lines highly expressing the anti-ganglioside GM3 human antibodies were successively expanded to obtain anti-ganglioside GM3 human antibody-expressing stable cell lines (CJ15, CJ25, CJ38, CJ45, and CJ67).

1.6 Measurement of IgM concentration in culture supernatants

IgM concentration in the culture supernatants was measured as described below. Anti-Human IgM (BIOSOURCE) was diluted using a coating buffer (0.1 M $NaHCO_3$ and 0.02% NaN_3) to prepare a 1 µg/mL solution. The diluted solution was added to a 96-well ELISA plate at 100 µL/well, and then reacted at 4°C for 24 hours or longer to coat the plate.

After washing the wells with Rinse Buffer, blocking was carried out by adding 200 µL/well of Diluent Buffer and reacting at room temperature for 1 hour or longer. Compositions of the Rinse Buffer and Diluent Buffer are shown below.

Rinse Buffer: PBS(-)

                              0.05% Tween20
                Diluent Buffer: 50 mM Tris,
                              1 mM MgCl₂,
                              0.15 M NaCl,
5   0.05% Tween20,
                              0.02% NaN₃,
                              1% BSA

Next, culture supernatant suitably diluted with Diluent Buffer was added to the wells at 100 μL/well, and allowed to react at room temperature for 1 hour. After washing with Rinse
10   Buffer, alkaline phosphatase-conjugated goat anti-human IgM (BIOSOURCE) diluted 4,000 times with Diluent Buffer was added at 100 μL/well, and reacted at room temperature for 1 hour. Finally, wells were washed with Rinse Buffer, and alkaline phosphatase substrate (SIGMA) was added thereto. The absorbance was determined at the 405 nm measurement wavelength and 655 nm reference wavelength using Benchmark Plus absorption spectrometer (BioRad). The
15   concentration of IgM was calculated by comparing with a purified anti-ganglioside GM3 human antibody (Hoon *et al.*, Cancer Research 1993; 53: 5244-5250).

Each type of stable cell line expressing anti-ganglioside GM3 human antibodies was cultured in a 75 cm²-culture flask at an initial cell density of 2 x 10⁵ cells/mL. The IgM concentration in the culture supernatants was measured by the method described above. The
20   results are shown in Table 2. The amount of IgM produced was approximately 20 mg/L on the third day and approximately 50 mg/L on the seventh day. The productivity indicating the production ability of a single cell was 5 to 19 pg/cell/day. Since IgM is a type of immunoglobulin that forms multimers, expression level of IgM in recombinants is low, and therefore, its large-scale preparation was considered difficult. However, the present results
25   showed that highly productive recombinant IgM-expressing cells can be produced from CHO cells.

Table 2

J-chain expression	Cell lines	Production amount after culturing for 3 days (mg/L)	Production amount after culturing for 7 days (mg/L)	Productivity (pg/cell/day)
Absent	CA02	24.1	36.9	14.1
	CA15	11.8	39.7	4.9
	CA19	27.1	62.3	13.1
	CA20	20.2	35.4	10.5
	CA24	25.0	41.5	10.7

Present	CJ15	29.4	N.T.	19.4
	CJ25	24.4	N.T.	18.1
	CJ38	14.9	N.T.	12.4
	CJ45	26.4	N.T.	18.7
	CJ67	18.0	N.T.	12.8

N.T.: Not Tested

Industrial Applicability

      The present invention enabled stabilization of highly concentrated proteins in solutions at low temperature. Since the present invention enables stable long-term storage at low temperature of pharmaceutical formulations comprising proteins such as IgM as an active ingredient, it can significantly contribute to particularly the preparation of protein formulations.